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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  A61K 37/00	<b>A1</b>	<b>(11) International Publication Number:</b> WO 93/14778  <b>(43) International Publication Date:</b> 5 August 1993 (05.08.93)
<b>(21) International Application Number:</b> PCT/US93/00492 <b>(22) International Filing Date:</b> 21 January 1993 (21.01.93)  <b>(30) Priority data:</b> 07/824,370                      23 January 1992 (23.01.92)      US  <b>(71) Applicant:</b> VICAL, INC. [US/US]; 9373 Towne Centre Drive, Suite 100, San Diego, CA 92121 (US).  <b>(72) Inventors:</b> RHODES, Gary, H. ; 1618 Burgundy Road, Leucadia, CA 92024 (US). DWARKI, Varavani, J. ; 4834 Gallatin Way, No. 8, San Diego, CA 92117 (US). FELGNER, Philip, L. ; HWANG-FELGNER, Jiin-Yu ; 5412 Las Palomas, P.O. Box 3392, Rancho Santa Fe, CA 92067 (US). MANTHORPE, Marston ; 12418 Kestrel Street, San Diego, CA 92129 (US).		<b>(74) Agents:</b> SIMPSON, Andrew, H. et al.; Knobbe, Martens, Olson & Bear, 620 Newport Center Drive, 16th Floor, Newport Beach, CA 92660 (US).  <b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EX VIVO GENE TRANSFER  <b>(57) Abstract</b>  A method for delivering a polypeptide to the interior of a cell of a vertebrate ex vivo is provided comprising the steps of: removing live cells from the vertebrate, contacting the cells with a preparation comprising an effective amount of polynucleotide operatively coding for the polypeptide and lipid effective to deliver the polynucleotide in the cells; and freezing or returning the cells to the vertebrate within 48 hours after the contacting step so that the cells express the polynucleotide in vivo. The method additionally can comprise substantially separating the cells from the surrounding extracellular matrix.		

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## EX VIVO GENE TRANSFER

Field of the Invention

The present invention relates to methods for delivering polynucleotide to the interior of a cell of a vertebrate ex vivo. In particular this invention is directed to rapid and convenient methods for introducing genetic material into cells, which may then be returned to the vertebrate. The genetic material may be introduced into the cell to correct a functional cellular defect, to augment an immune response or to express a foreign protein.

Background of the Invention

Gene transfer is a term that broadly encompasses methods for the introduction of gene sequences into a cell or group of cells. There are a variety of methods currently in use to introduce exogenous genetic material into cells. These methods include calcium precipitation, viral vector-mediated delivery, electroporation and liposome-mediated delivery.

Gene transfer in vitro is used to study the effect of a given gene and its resulting gene product on a given population of cells. Early work in the field of gene transfer was directed to the areas of gene replacement and augmentation. Gene augmentation includes approaches for introducing a correct copy of a mutated gene into defective cells or a copy of a foreign sequence for gene expression within that cell. Gene replacement includes approaches to correct defective genetic sequences through site-specific recombination, thus permitting targeted homologous recombination for a known gene sequence. It is generally assumed that both approaches require the stable expression of exogenous polynucleotide sequences.

Following transfection, cells stably expressing the polypeptide encoded from the transfected gene are selected for further analysis. Examples of stable transfectants selected for gene therapy directed to skeletal muscle and hematopoietic cells are found in publications by Salminen et al. and Dick et al. (Hum. Gene Ther. 2:15-26, 1991 and Blood 78:624-634, 1991

respectively) which are hereby incorporated by reference. Stable expression is thought to require either stable integration or homologous recombination of DNA into the host chromosome, although there is some evidence that  
5 polynucleotide formed as episomal-like structures may be able to direct the stable expression of polypeptide as well.

There are a variety of methods, known to those with skill in the art, for introducing genetic material into a host cell. Viral vectors encapsulated in virus particles are among the  
10 more efficient methods for gene delivery. Many viral vectors require gene integration for replication and gene expression. However, since viral vectors do integrate into the host genome with some efficiency, they may potentiate harmful recombination events that promote oncogenesis. Other methods  
15 of polynucleotide delivery include liposome-mediated transfection and the introduction of naked polynucleotide into tissue. Irrespective of the method for gene delivery, polynucleotide integration into the host genome can be harmful. Therefore stable transfection involving integration  
20 into the host genome will increase the probability of harmful recombination events.

A major challenge to gene therapy is the effective delivery of the gene sequence to the targeted tissue. The efficacy of gene therapy for in vivo applications is limited  
25 by the accessibility of the gene to the target cells. Viral vectors can be directed to specific cell types by incorporating cell surface binding proteins into the viral package. Similarly, amphotropic viral vectors or liposomes can be used to deliver gene sequences to a variety of cell  
30 types. While vectors can be introduced into the body through oral or parenteral routes, the vascular endothelium and mucosal membranes are barriers to vector dissemination. The introduction of therapeutic genes directly into a tissue site may be more beneficial to a patient than methods relying on  
35 dissemination through oral and parenteral entry routes.

Since viral vectors require cell replication for gene expression, liposome-mediated delivery is better suited for

some cell types. Vectors can be injected into the tissue of interest, but experimental results indicate that the expressed protein is concentrated within those cells immediately juxtaposed to the needle tract. Cells removed but still proximate to the tract show reduced to insignificant levels of gene expression. Gene therapy would be more effective if gene expression could occur in a radius wider than cells directly adjacent to the needle tract.

An alternative to direct in vivo gene delivery for gene augmentation or gene replacement involves removing cells from a patient, optionally expanding these cells in culture, transfecting the cells and identifying stable transfectants. Following identification of stable transfectants, the cells are optionally expanded and returned to the host. This procedure has been used most successfully to transfect cells of hematopoietic origin.

Other cell types have been stably transfected in culture including fibroblasts, myoblasts and hepatocytes. Stable cell lines have been used experimentally for certain applications in place of autologous host cells. These procedures require that the cells are maintained in culture. Thus one of the major drawbacks of in vitro expansion before transfection and the selection of stably transfected cells following transfection is the length of time the cells spend in culture. The time required to obtain cells from a patient, treat the cells, select stable transfectants and return these to their host can be a matter of weeks to months. In the absence of the in vivo cellular milieu, protein expression changes. With extended time in culture, certain subpopulations of cells survive under selection conditions and replicate better than others. The conditions used to select stable transfectants also selects for cells able to survive in culture. Cell survival in vitro does not translate into improved cell survival in vivo. Thus cells adapted to culture or cells expanded in culture may not be able to survive in vivo. A method that minimizes cell time in culture will reduce the likelihood that cell changes occur in vitro.

Current gene therapy procedures are labor intensive and require a high level of technical expertise. Thus, gene therapy is typically restricted to large medical research centers with highly sophisticated technical support. Individuals lacking access to these centers are not likely to benefit from technically intensive genetic therapies. A procedure to introduce a gene of interest into host cells with minimum turn around time, that could be performed rapidly and routinely at a doctor's office, a medical clinic or local hospital would allow greater numbers of people to benefit from gene therapy.

The method disclosed herein permits cells from a given tissue to be processed, treated with the desired gene and returned to the patient in a timely manner. Cell replication and selection are not required. Thus the level of technical skill, the time and the cost of the gene therapy procedures is reduced.

#### Brief Description of the Figures

Figure 1: Three-dimensional bar graph of results from the microtiter transfection procedure.

Figures 2a-2d: Three-dimensional bar graphs comparing cell density to transfection efficiency.

Figure 3: Graph of IgG antibody titer to HIV gp120 immunized cells ex vivo.

#### SUMMARY OF THE INVENTION

The present invention provides a method for delivering a polypeptide to the interior of a cell of a vertebrate ex vivo comprising the steps of removing live cells from the vertebrate, contacting the live cells with a preparation comprising an effective amount of polynucleotide operatively coding for the polypeptide and lipid effective to deliver the polynucleotide into the cells, and returning the cells to the vertebrate within 48 hours after the contacting step so that the live cells express the polynucleotide in vivo. Unlike prior art ex vivo methods, the present invention does not require post-transfection selection and expansion steps.

Thus, the entire procedure can be completed in a number of hours, instead of weeks.

Another aspect of the present invention is a method for preparing transfected cells for use expressing a polypeptide in a vertebrate, comprising the steps of removing live cells from the vertebrate, and transfecting at least some of the live cells by contacting them with a preparation comprising an effective amount of a polynucleotide operatively encoding the polypeptide and a transfection-facilitating lipid, the preparation being effective to deliver a substantial amount of the polynucleotide into the cells by contact therewith within 48 hours, and suspending the transfected cells in a pharmacologically acceptable carrier for introduction into the vertebrate, in the absence of a selection step to separate or expand the transfected cells. Preferably, the removing step additionally comprises substantially separating the cells from the surrounding extracellular matrix. Furthermore, if one desires to store the cells (instead of immediately using them), the method may include freezing the cells within 48 hours after the contacting step. In one embodiment, the peptide is adapted to treat a disease of the vertebrate caused by a functional gene deficiency. In most cases, the polynucleotide is adapted to express the polypeptide in the live cells. The expression of the polynucleotide by the cells may be transient, or may persist for a substantial length of time. In one preferred embodiment, the polypeptide is an immunogenic polypeptide in the vertebrate. In such a case, the vertebrate (which may be a mammal, including a human, a bird, or some other vertebrate) develops an immune response against the immunogen after the transfected cells are reintroduced. This method may be used to immunize the animal. In one preferred embodiment, the operatively codes for a lymphokine. Cells that may be used include (for example) white blood cells, myoblasts, and bone marrow cells.

The present invention includes a medicament prepared in accordance with any one of the foregoing methods. Also included in the present invention is a method for delivering

a polypeptide to a vertebrate *in vivo*, comprising the step of injecting or implanting such a medicament into a vertebrate. The cells used in this method preferably include cancer cells, white blood cells, bone marrow cells including stem cells and myoblasts. Where the method is applied to cancer cells, the polynucleotide preferably operatively encodes lymphokine.

#### DETAILED DESCRIPTION OF THE INVENTION

The methods disclosed teach a procedure to remove a tissue or cell sample from a patient, contact the cells of the sample with the desired gene ex vivo and return the cells to the patient preferably within less than 40 hours after removing the sample from the patient. The procedure delivers a polynucleotide operatively coding for a polypeptide to the interior of a cell of a vertebrate. The term "polypeptide" and "protein" are used interchangeably within this patent application. The term "exogenous polypeptide" is used to differentiate protein naturally occurring in the cell from protein introduced into the cell using the methods of this invention. The term "removing" is used to describe any method known to those with skill in the art to obtain a sample of live cells from a vertebrate patient. Methods to remove a live cell sample include but are not limited to venipuncture, cell scraping, and biopsy techniques that include punch biopsy, needle biopsy and surgical excision. The term "contacting" includes methods to introduce polynucleotides into a cell. These methods include transfecting methodologies including electroporation, liposome-mediated transfection, calcium phosphate precipitation and DEAE-dextran. The term "returning" includes methods known to those with skill in the art to replace cells in the body. These include, but are not limited to, intravenous introduction, surgical implantation and injection.

In another embodiment of this invention, a method is disclosed that comprises removing live cells from a vertebrate patient, substantially removing the cells from the surrounding



extracellular tissue, contacting the live cells with a preparation comprising an effective amount of polynucleotide and returning the cells to the patient.

5 In the process of the present invention, the cells are isolated, exposed to the gene of interest, and returned directly to the host with minimal time in culture to reduce the potential for cell change in vitro. Some of the cells express the desired gene transiently, others stably; however, with a minimum turn around time, the cells are more likely to survive in vivo. Whether stable or transient expression is achieved; the result is beneficial. If needed, the therapy can be repeated to maintain the desired level of exogenous gene expression. Transient gene expression in vitro is generally defined as temporary gene expression that diminishes over time under selective conditions. In vivo transient expression can more broadly be defined as gene expression occurring over periods of less than one year to periods as short as one week or one month. The gene therapy application, the vector construct, whether or not chromosome integration has occurred, the cell type and the location of cell implantation following transfection will all influence the length of time that a particular gene is expressed. The methods discussed herein teach one of skill in the art to introduce gene sequences such that a variety of cells from a variety of tissues can be treated ex vivo and rapidly returned to a host for gene expression without selection for stable transfectants.

20 This invention has a number of applications, some of which will be discussed in detail below. The therapy can be used to express exogenous polynucleotide in a variety of tissues not readily accessible by other gene therapy means. For example, liver cells can be transfected with the LDL receptor to reduce serum cholesterol in vivo, and muscle can be removed to treat the cells with the defective gene product involved in Duchennes muscular dystrophy. Progenitor cells from the hematopoietic system can be treated at a predifferentiated stage to correct hereditary disorders such

as beta-thalassemia. The method is contemplated to be particularly useful for the treatment of diseases due to a functional gene deficiency. For these applications, the polynucleotide encoding exogenous polypeptide codes for the polypeptide deficient or malfunctioning in that particular genetic disease.

The transient expression of the gene product can be advantageously used to promote an immune response. Thus viral diseases can be treated by interferon expression and cytokines can be used to stimulate the immune system to react against foreign antigens or cancers. Similarly, foreign protein can be expressed transiently from target cells to generate an immune response.

#### Biopsy and Tissue Processing

A biopsy or cell sample is preferably obtained from a patient in need of gene therapy. However it is additionally contemplated within the scope of this invention that the treated cells could be derived from a secondary source such as a cell line or a cell donor. The cell sample is preferably obtained from a tissue where gene expression would be most advantageous. The cell sample may be derived from a variety of tissues including skin, liver, pancreas, spleen, muscle, bone marrow, nervous system cells, blood cells, and tumor cells.

The type of tissue and location of that tissue will suggest the best method of tissue sampling. Those with skill in the art will readily appreciate the variety of biopsy and sampling procedures available for a given tissue type. Biopsy of formed tissue may be obtained by needle, catheter, punch biopsy or surgical excision. For example, a skin biopsy may be best obtained by a skin punch, while a liver biopsy may be best obtained by a needle biopsy.

The size and quantity of biopsy is determined by the level of cell recovery following tissue processing. It is estimated that the transfection procedure can be optimized for the transfection of as little as 50-100 cells. The biopsy size should be adjusted to account for cell recovery. A skin

biopsy of 0.5 cm<sup>2</sup> should produce a minimum of 2500-5000 cells. Low cell recovery from a given biopsy may necessitate a larger biopsy size or a change in the cell dissociation procedure for a given tissue.

5           For cell types present in the blood, a simple blood draw into a heparinized, EDTA, or citrate tube can be processed by centrifuging at 1000 rpm for 10 min. The buffy coat is drawn into a Pasteur pipette and the cells contained therein are washed to remove serum, red blood cells, and debris. These  
10 cells are ready for gene therapy.

          In one embodiment of the present invention, the cells are separated from surrounding supportive tissue or extracellular matrix. As one example of a cell separation method, a skin biopsy is enzymatically digested to dissociate the cells from  
15 their extracellular matrix. The following procedure is only one of many procedures for tissue dissociation. This particular procedure is useful for skin, kidney and lung tissue dissociation.

          After the biopsy is removed from the patient it is placed  
20 in sterile saline and washed to remove blood and debris. The tissue is minced with a scalpel or single edge razor blade, in a small amount of Hanks Balanced Salt Solution (GIBCO, Grand Island, New York). This permits the relative pH of the tissue sample to be monitored during processing. Maintaining  
25 physiologic pH is important to cell survival. The mincing is performed for approximately 10 minutes or until the tissue resembles a thick paste. The paste is transferred to a small Erlenmeyer flask or beaker (5 ml.) and the volume of cell paste is approximated. Three volumes of a mixture of 0.25%  
30 trypsin/ 1mM EDTA (GIBCO) and 1% wt/vol. collagenase B (Boehringer Mannheim, Indianapolis, Indiana) is added to the cell paste. The flask is covered with foil and placed on a rotational platform in a 37° C incubator for 15-30 min. Alternatively, a sterile small magnetic stir bar is added to  
35 the sample and the flask is placed on a magnetic stir plate. The sample is monitored every 10 min both to maintain the pH

and to monitor tissue dissociation. At the end of the incubation period, fetal calf serum or albumin is added, and the sample is passed through three layers of sterile cheese cloth to remove connective tissue fragments. The liquid that  
5 passes through the cloth is checked for visible cell clumps. If a large number are present, the clumps are spun down (1200 rpm, 5 min) and the trypsinization/collagenase procedure is repeated. The enzymatic solution is washed away from the cell suspension and the cells are washed and transferred into the  
10 appropriate size vessel for transfection.

Methods for the dissociation of other tissues may employ different enzyme preparations. Other enzymes used in tissue dissociation include Versene, pancreatin and other  
15 collagenases. Biopsy samples from tissues that have minimal connective tissue will not require collagenase treatment. For these samples, trypsin digestion may be sufficient. For samples from other tissues, trypsin mediated dissociation may be too harsh. Here, EDTA or other chelators will sufficiently  
20 dissociate the tissue while maintaining cell survival. For still other tissue samples, mincing or aspiration may generate a cell suspension without enzymatic treatment. For some applications, tissue dissociation may not be required; the biopsy sample is transfected directly without further manipulation.

25 For some gene therapy applications, a uniform cell population is preferred. The cells are first dissociated from a biopsy or blood sample and then further separated from other cell types. There are a variety of methods known to those with skill in the art for removing a particular cell type from  
30 a mixed suspension of cells.

Cell types can be differentiated from one another by their density or size. For example, mesh screens, Ficoll or sucrose gradient centrifugation can be used to separate one  
35 cell population from another. Cells can additionally be separated on the basis of their adherent properties. For example, fibroblasts will adhere and settle onto plastic more quickly than epithelial cells. Populations of cells can also

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be separated from one another by virtue of surface protein. Lectins or antibodies can be used to selectively separate one population of cells from another either using panning (passing cells over a surface with bound lectin or antibody), column chromatography or fluorescent activated cell sorting. According to a preferred method, cells from a blood sample are separated in a device (The Collector, Applied Immune Sciences, Menlo Park, California) which comprises multiple polystyrene plates to which monoclonal antibodies, which bind selectively to specific cells, have been permanently attached. When a blood sample is processed in The Collector, targeted cells remain attached to the polystyrene surface, while other cells pass through the device. The captured cells can then be released by mechanical or chemical means. Those with skill in the art will be able to select the appropriate cell separation method for their particular application.

The washed and dissociated cells can be stored for up to 24 h or longer in culture. However, the cells are preferably transfected immediately following tissue dissociation and cell purification. If there is a delay between cell isolation and transfection it is worthwhile to return the cells to a nutritive environment. The cells can be stored in tissue culture medium with a composition compatible to the particular cell type. Typically Eagles (EMEM) or Dulbecco's minimum essential medium (DMEM) with 10% fetal calf serum (FCS) or fetal bovine serum (FBS) and an antibiotic solution (Fungi Bact Solution, Irvine Scientific, Irvine, California; contains penicillin, streptomycin and fungizone) are used to maintain the cells ex vivo.

#### Polynucleotide Preparation

The invention disclosed herein can employ either DNA or RNA. DNA delivery may be preferred when stable or more prolonged gene expression is favored. However, either RNA or DNA gene delivery can be used for transient gene expression applications. As noted, the method makes no selection for

transient versus stable expression. However, without selection, it is likely that the majority of the cells will be transiently transfected. Transient expression permits gene expression for periods of hours or months. While repeated  
5 therapies may be required for some applications, the patient nevertheless benefits from the effect of gene expression. Like a drug, the dosage of polynucleotide and the number of delivery sites to the tissue can be monitored and adjusted accordingly.

10 DNA encoding the therapeutic gene may be circular or linear and should contain regulatory elements that facilitate expression in the target cells. Current progress in molecular biology is directed to the identification and isolation of tissue specific promoters. As these tissue specific promoters  
15 are identified, they may be incorporated into the gene expression vector and used for ex vivo gene therapy strategies in a particular tissue. Similarly more promiscuous promoters may be selected for a given application based on their promotional strength. Thus the actin promoter, the Rous  
20 Sarcoma virus (RSV) promoter or the Myo D promoter may be used for myoblast and muscle transfection. The cytomegalovirus (CMV) immediate early (IE) gene/enhancer (Foecking et al., Gene 45:101 (1986) hereby incorporated by reference), the RSV promoter/enhancer (Wolff, J.A. et al., Science 247: 1465-1468,  
25 hereby incorporated by reference), the  $\beta$ -actin gene/promoter enhancer (Kawamoto et al, Mol. Cell. Biol. 8:267 (1988), incorporated by reference) and the  $\alpha$ -globin promoter (Mellon, P., et al., Cell 27:279-288 (1981) also incorporated by reference) can be used for more ubiquitous cell expression.  
30 It is additionally contemplated that the CMV IE gene/enhancer-promoter with the first intron, (Chapman, B.S. et al., Nucleic Acids Research 19:3979-3986, hereby incorporated by reference) and the MCK promoter (Jaynes, et al., Mol. Cell. Biol. 8:62 (1988), hereby incorporated by reference) may be used for  
35 transfection in myoblasts followed by intramuscular injection for expression in mature muscle fibers. The transfected DNA

should additionally contain regions to mediate ribosome binding, polyadenylation signals and include enhancer regions to facilitate in vivo gene expression.

RNA encoding the polypeptide of interest should similarly contain appropriate promoter elements and ribosome binding sites. Preferably, the RNA is capped or stabilized to promote translation and to minimize exonuclease activity. The use of RNA in cationic-mediated gene transfer is described in a publication by Malone et al. (Proc. Natl. Acad. Sci. USA 86:6077-6081 (1989), hereby incorporated by reference). Alternatively, the mRNA can include particular 5' sequences that permit expression of uncapped mRNA. For example, some members of the picornavirus family including encephalomyocarditis virus (EMCV) have mRNAs that are naturally uncapped, yet they are translated and stable. A 650 bp (base pair) sequence in the 5' untranslated region provides a ribosome binding site that permits ribosome binding without a capping structure (Elroy-Stein, et al. Proc. Natl. Acad. Sci. USA 86:6126-6130 (1989), hereby incorporated by reference). These sequences can be used as replacements for the cap structures.

If calcium phosphate precipitation or electroporation is used to transfect the cells, additional precautions may be required to minimize RNase contamination of reagents during the transfection process.

#### Liposome mediated transfer of polynucleotide sequences

The "contacting" step of this invention includes methods for introducing polynucleotide sequences into target cells. The methods include calcium phosphate precipitation, DEAE-dextran, electroporation and liposome-mediated gene transfer. These methods are well known to those with skill in the art.

In a preferred embodiment, liposome-mediated gene transfer is used to introduce the polynucleotide into the target cells. Liposomes comprise amphipathic lipids forming hollow lipid vesicles. They have been used in vitro as a mechanism for introducing genetic sequences into tissue culture cells (Mannino, R.J., Fould-Fogerite, S.,

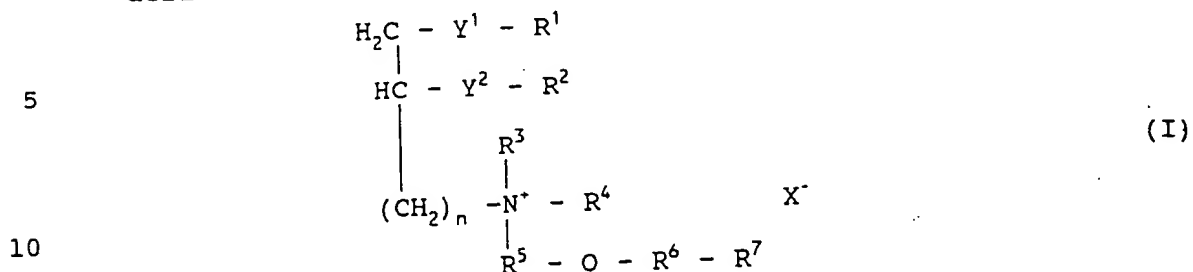
Biotechniques 6:682-690, 1988). Lipids forming liposomes may be positively charged (cationic), negatively charged (anionic) or neutral. A major advance in liposome development was the discovery that a positively charged synthetic cationic lipid, N-[1-92,3-dioleyloxy)propyl]-N,N,N-trimethylammoniumchloride (DOTMA) could interact spontaneously with DNA to form lipid-DNA complexes capable of fusing with negatively charged lipids associated with cell membranes (Felgner, P.L. et al., Proc. Natl. Acad. Sci.(USA) 84:7413-7417 (1987)) and U.S. Patent No. 4,897,355 to Eppstein, D. et al). Liposome-mediated gene delivery, unlike retroviral-mediated gene delivery, can deliver either RNA or DNA. Thus DNA, RNA, a modified polynucleotide or a combination thereof can be introduced directly into the cell cytoplasm (Malone et al. Proc. Natl. Acad. Sci.(USA) 86:6077-6081, 1989).

Lipofectin™ (GIBCO/BRL, Gaithersburg, Maryland) is used in vitro for nucleic acid transfection procedures. Lipofectin™ consists of 0.5 mg/ml DOTMA and 0.5 mg/ml dioleoylphosphatidyl ethanolamine in sterile water. DOTMA, the cationic lipid component of Lipofectin™, is a diether lipid. Other suitable cationic lipid species comprise known cationic lipids, such as 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) or N-(w, w-1-dialkoxy)-alk-1-yl-N, N, N-trisubstituted ammonium surfactants, or complex cationic lipids having similar structures and properties or mixtures of these.

Particularly preferred cationic lipids including those cationic lipids postulated to be more readily degradable in vivo are those disclosed in a co-pending U.S. Application No. 686,746 filed April 19th, 1991 by Felgner et al., which is hereby incorporated by reference. These include analogs of DORI (DL-1,2-dioleoyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium) and DORIE (DL-1,2-O-dioleoyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium) as well as DORI ester/ether compounds (DL-1-O-oleyl-2-oleyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium or DL-1-oleyl-2-O-



oleyl-3-dimethyl-aminopropyl- $\beta$ -hydroxyethylammonium) or other derivatives having the general formula



wherein

$\text{Y}^1$  and  $\text{Y}^2$  are the same or different and are  $-\text{O}-\text{CH}_2-$ ,  $-\text{O}-\text{C}(\text{O})-$ , or  $-\text{O}-$ ;

$\text{R}^1$  and  $\text{R}^2$  are the same or different and are H, or  $\text{C}_1$  to  $\text{C}_{23}$  alkyl or alkenyl;

$\text{R}^3$  and  $\text{R}^4$  are the same or different and are  $\text{C}_1$  to  $\text{C}_{24}$  alkyl, or H;

$\text{R}^5$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl straight chain or branched chain;

$\text{R}^6$  is  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$ , a diaminocarboxylic acid which is alkyl, aryl, or aralkyl, or  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$  linked

to said diaminocarboxylic acid, or is absent;

$\text{R}^7$  is H, spermine, spermidine, a histone, or a protein with DNA-binding specificity, or wherein the amines of the  $\text{R}_7$  moiety are quaternized with  $\text{R}^3$ ,  $\text{R}^4$ , or  $\text{R}^5$  groups; or

$\text{R}^7$  is an L- or D-alpha amino acids having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine, ornithine or derivatives thereof, or the same amino acids wherein the amine of the  $\text{R}_7$  moiety is quaternized with  $\text{R}^3$ ,  $\text{R}^4$  or  $\text{R}^5$  groups; or

$\text{R}^7$  is a polypeptide selected from the group consisting of L- or D-alpha amino acids, wherein at least one of the amino acids residues comprises arginine, histidine, lysine, ornithine, or derivatives thereof;  $n$  is 1 to 8;

$m$  is 1 to 18; and

$\text{X}$  is a non-toxic anion.

Non-toxic anions described herein may be those of pharmaceutically non-toxic acids including inorganic acids and organic acids. Such acids include hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, benzoic, citric, glutamic, lactic acid and the like. For the preparation of pharmaceutically acceptable salts, see S.M. Bēge et al., Journal of Pharmaceutical Sciences, 66:1-19 (1977). Further, DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium) could be used in combination with the improved cationic lipids or in combination with cholesterol, lyso lipids or neutral lipids.

In addition to these cationic lipids, other lipids may be added to the cationic lipid of choice. These include but are not limited to lyso lipids of which lysophosphatidylcholine (1-oleoyllysophosphatidylcholine) is an example, cholesterol, or neutral phospholipids including dioleoyl phosphatidyl ethanolamine (DOPE) or dioleoyl phosphatidyl choline (DOPC). The ratios of lipids may vary to include a majority of cationic lipid in combination with cholesterol or mixtures of lyso or neutral lipids. Preferred lipid formulations will be detailed in the examples below.

As a preferred example of the transfection procedure, cationic lipid-mediated DNA transfection is discussed. Examples of cationic lipid-mediated transfection relating to specific applications of this invention are discussed in Examples 1 and 2.

Lipofectin<sup>TM</sup> is a commercially available cationic lipid. For those formulations that are not commercially available or are not formulated into liposomes, the lipid molecules and/or other lipid components are solubilized in chloroform and dried overnight with a vacuum pump to remove trace amounts of chloroform. The lipid film is next hydrated with sterile purified distilled deionized water and the sample is sonicated at 10°C to clarity. Mixtures of lipid are combined with polynucleotide for transfection. These techniques are known to those with skill in the art.

As mentioned, there are a variety of tissue dissociation methods available along with different cell purification schemes. Biopsies and cells may be frozen before or after tissue dissociation. Dissociation and purification procedures should be performed as rapidly and expediently as possible after biopsy to maximize cell viability. Once dissociated, the cells are transfected. The transfected cells are preferably returned immediately to the patient without expansion or selection in culture. For some applications the biopsied tissue can be dissociated, treated with the desired polynucleotide, and frozen without expansion or selection in culture. Samples can be thawed as necessary for future treatments. Similarly, a portion of the frozen cells can be thawed and assayed for transfection efficiency or for gene expression. There are a variety of methods known to those with skill in the art for freezing cells. Standard methods for freezing cells involve freezing cell suspensions in 5-20% FCS with 2-7% DMSO or glycerol. The cells are placed at -70°C overnight and stored at or near -120°C. Modifications of this standard technique are well known to those with skill in the art.

In a preferred embodiment of the invention it is contemplated that the ex vivo procedure can be used to activate the immune system. An effective amount of polynucleotide is delivered to the appropriate cells and expression of the polypeptide encoded by the polynucleotide results in activation of the immune system.

This particular strategy has a variety of uses. The most direct use is to stimulate the humoral and cellular immune system. A gene operatively encoding a polypeptide of interest is introduced into a population of cells obtained from a blood sample or tissue sample. These cells are returned to the patient. The expression of a foreign gene in the host stimulates cell presentation to the immune system resulting in a humoral response from B lymphocytes and a cellular response in the T cell population. In addition, expression of a

foreign gene in a given population of immune system cells can directly stimulate immune responses.

5 The data in Example 5 and 6 illustrate an approach to the activation of the immune system which allows better control over cellular immune responses against virus infected cells and cancer cells in vivo than traditional Immunization protocols. A growing body of data indicate that intracellular antigen synthesis is required for optimal presentation of antigens to the cellular arm of the immune system. This is because antigen presentations in the context of major histocompatibility complex (MHC) class I protein is best achieved when antigen is processed together with MHC class I protein in the Golgi apparatus. The benefit of intracellular antigen synthesis to the potency of an immune response helps to explain the difficulties associated with the generation of functional cellular immune responses against recombinant antigens by conventional systemic, extracellular injection of subunit vaccines. Consistent with this view, it has been shown that retrovirus transformed primary cells or syngeneic cells lines, expressing intracellular antigen, can present antigen in vivo and stimulate cellular immune responses in an MHC-I restricted manner. Our results indicate that the non-retroviral mediated approach can similarly bypass this restriction, and we anticipate that it will be preferred, for regulatory reasons, to approaches which require viral vectors. This ex vivo gene therapy will be a useful technique for generating an immune response. Transient expression of therapeutic polypeptide, as contemplated within the scope of this invention, is employed to transiently introduce gene product into the body as a therapeutic drug.

30 Another embodiment of the invention involves the stimulation of the immune system to recognize a given population of cancer cells as foreign. In this application, tumor cells are removed from a patient, transfected with a gene, and the cells are returned to the patient within 48 hours following transfection. In a preferred embodiment, the cells are immediately returned to the patient. The immune

system then recognizes those particular cancer cells expressing the exogenous polypeptide as foreign. Stimulation of an immune response in the general locale of the cancerous tissue increases the likelihood that untreated tumor cells will additionally be recognized as foreign, thereby generating generalized tumor regression. A number of immunogenic proteins could be used to stimulate the immune system toward cancerous cells. Both endogenous genes, such as those of the major histocompatibility complex, and foreign antigen may be useful.

A further candidate for immune system activation is the introduction and expression of lymphokine in the tumor cells or in other selected cells of the body. Lymphokines in general, and IL-2 in particular, are potent immune system stimulators. Lymphokine expression can be used to activate endogenous lymphokine activated killer cells (LAK cells). IL-2 stimulates the differentiation of null cells to become LAK cells. These cells are known to attack a variety of cancers and stimulation of their cell killing activity is enhanced by exogenous IL-2. A second cell type, tumor-infiltrating lymphocytes (TIL), is also stimulated by IL-2 and has been found to be more effective than LAK cells in promoting tumor regression. In addition, other cytokines are identified that are directly cytotoxic to tumor cells. Expression of these proteins in and around a tumor site results in tumor regression. Improved regulation of cytotoxic T-cells may also increase the clearance of latent viral infections and facilitate the destruction tumorigenic foci. Improved regulation of helper and suppressor T-cell populations by this approach may ultimately lead to improved methods for controlling autoimmune disorders including rheumatoid arthritis.

In another preferred embodiment of this invention, a muscle biopsy is used to isolate muscle myoblasts. Patient myoblasts can be isolated following the techniques of Blau et al. (Proc. Natl. Acad. Sci. USA 78: 5623-5627, 1981, which is hereby incorporated by reference). Myoblasts are readily

obtained from muscle without substantial inconvenience to the patient. A small amount of tissue, processed according to the techniques of Blau et al., yields sufficient quantities of myoblasts for transfection. The muscle myoblasts are then  
5 transfected ex vivo following the methods disclosed herein and returned within 48 hours after transfection to the muscle or to other tissues of the patient. Myoblasts can additionally be stored or frozen for a time without a significant loss in cell viability or a significant change in cell phenotype.

10 When myoblasts are reintroduced back into muscle, they are capable of fusing with the available mature myofibers. Thus myoblast delivery is a particularly efficient method for gene delivery to muscle cells. Since the myoblasts are readily isolated and conveniently obtained from muscle tissue  
15 of the patient, there is no risk of tissue rejection. Transient gene expression is more likely to be sustained over time from repeated therapies in autologous cells. Another important advantage to myoblast transfection is that muscle tissue is accessible to injection and underlies most surfaces  
20 of the body, thus the transfected cells can be injected in multiple locations as often as needed. Using muscle specific promoters like MCK (Jaynes et al., supra), gene product expression can be restricted to mature muscle fibers. The muscle cells thus express the desired polynucleotide sequence.  
25 Recent work indicates that stable myoblast transfectants injected into muscle tissue express their gene product into the blood stream (Hoffman, M. Science 254: 1455-1456, 1991).

It is also contemplated within the scope of the invention  
30 that any number of therapeutic agents could be used to treat the cell sample from a patient ex vivo. Just as polynucleotide could be transiently expressed from the isolated cells, any number of agents could be added to the cells to provide a transient therapeutic effect. Thus  
35 polynucleotide nucleoside analogs, riboenzymes, antisense polynucleotide, or other pharmaceuticals can be delivered to the cells.

Reintroduction of treated cells

Once transfection is complete, the cells are washed, concentrated and returned to the patient. The cells may be returned directly to their tissue of origin, or for some applications, the cells can be returned to a second location either within the tissue of origin or at a location distant from the tissue of origin. A large bore needle (18 gauge or larger) is preferably used to return cells to the body. Any bore size may be used that does not destroy the cells or restrict cell passage into the tissue. It may be beneficial to introduce the treated cells into multiple locations within a given tissue. For example in Duchenne's muscular dystrophy, multiple injections of treated cells will advantageously disperse the gene product throughout the tissue.

If the tissue has been treated with cationic liposome and polynucleotide without tissue dissociation, then the intact biopsy may be replaced by surgical implantation. Other cells, such as hepatocytes, can be introduced back into their organ of origin by injection into a vein leading into that organ.

Methods to return treated cancer cells to a patient will depend on the cancer type. Injection of autologous transfected cancer cells into either a tumor mass or bone marrow is possible. Expression of an MHC protein, a foreign protein or an immune stimulating protein can stimulate immune responses directed to both the treated and untreated tumor cells, thereby reducing or clearing the tumor mass. Other transfected cancer cells of hematopoietic origin may be introduced directly into the blood stream. Cells from hard tissue tumors may be replaced directly into a tumor mass to elicit tumor regression. This may be performed through external injection or internal injection using catheters or the like. In a preferred embodiment of this invention, the reagents necessary to perform the gene therapy are provided in kit form. Thus the procedures that have been outlined in Example 1 and 2 for optimization of gene transfection into a particular cell type are used to determine the optimal combination for transfection of a particular gene into a cell

obtained from a particular tissue. A transfecting dose of the gene is supplied as either RNA or DNA complexed to or separate from the cationic lipid or lipid transfecting reagent. Equipment required for a given therapy may additionally be supplied, including biopsy tool, balanced salts, medias, wash buffers, and tissue dissociation tools. Thus the optimized procedure is reduced to a routine laboratory procedure requiring minimal laboratory manipulation. The technician receives a biopsy from the physician, a blood sample or a skin punch. The sample is processed according to kit directions. The appropriate number of cells for the particular regime (as optimized) is placed into transfection wells and transfected with the gene of interest. Following an incubation and wash period the cells are inserted, preferably by injection or surgical implantation, back into the patient.

Standard methods for cell transfection require in excess of 30,000 cells per transfection. Using this technology, a biopsy or cell sample must first be expanded in culture to provide a sufficient target population for transfection. Using the methods disclosed herein, efficient transfection and gene expression is possible using cell numbers easily isolated from a standard biopsy or blood draw. In addition, earlier methods required that the technician select those cells stably expressing the gene of interest following transfection and these cells were further expanded. The invention disclosed herein does not require cell expansion prior to transfection nor cell selection following transfection. Cells are immediately frozen or returned directly to the patient. This reduces the required technician time and expertise such that gene therapy can become a widely available medical procedure.

Unlike conventional gene therapy, in which stably transformed genes manufacture a relatively constant level of gene product for longer periods of time, transient expression is often desirable in the practice of the present invention. This major conceptual difference from the prior art affectively permits the physician to use the gene of interest as a drug. Thus a gene can be administered periodically; the



dosage can be adjusted, and the effect is ultimately transient. None of these benefits are available through conventional gene therapy.

5           Particular embodiments of the invention will be discussed  
in detail and reference will be made to possible variations  
within the scope of the invention. There are a variety of  
alternative techniques and procedures available to those of  
skill in the art which would similarly permit one to  
10           successfully perform the intended invention.

#### EXAMPLE 1

##### 15           Microtiter Plate Transfection Protocol

Methods for optimizing the transfection protocol are  
provided below. Each gene therapy regime should be optimized  
to facilitate maximum transfection efficiency. Once the  
optimum conditions are obtained for a particular cell type,  
20           cationic lipid reagent and polynucleotide, then individual  
wells containing as few as 50-100 cells can be transfected.  
The optimization scheme provided below uses 5,000 to 40,000  
cells per well but can be scaled down for optimization of  
techniques employing fewer cells.

##### 25           Cationic Lipid Dilution Scheme

20,000-40,000 cells/well were seeded onto a 96-well  
micro-titer plate and incubated overnight at 37° C in a  
humidified, 5 to 10% CO<sub>2</sub> environment. DNA and cationic lipid  
serial dilutions were prepared in two separate 96-well plates  
30           according to the following scheme.

## Cationic Lipid Dilution Scheme

2x Serial Dilution in Opti-MEM Columnwise

Cationic Lipid (nmole)

5

16.8      8.4      4.2      2.1      1.05      0.53      0.26      0.13

10

15

X									X		
X											
X											
X											
X											
X											
X											
X											

20

Dilute cationic lipid vesicles to 0.747 mM with water.

Disperse 66 ul of Opti-MEM per well in Column 1 (A1..H1) and A10.

Disperse 60 ul of Opti-MEM in each well (A2.H8 and B10.H10).

Load 54 ul of 0.747 mM cationic lipid to each well of column 1 (A1..H1) and well A10 marked as "X".

Transfer 60 ul to next column after mixing.

25

DNA Dilution Scheme

2X Serial Dilution in Opti-MEM Row-wise

DNA  
(nmole)-DNA  
Ctrl

30

35

6.06	o	o	o	o	o	o	o	o	o			
3.03												
1.52												
0.76												
0.38												
0.19												
0.095												
0.047												

40

Dilute DNA prep to 80 ug/ml with Opti-MEM (avg nucleotide MW=330)

Dispense 70 ul of Opti-MEM in each well

Load 70 ul of 80 ug/ml DNA per well to the first 9 wells of row 1.  
Transfer 70 ul to next row after mixing to prepare serial dilutions.

5

Equal volumes of DNA were transferred to the corresponding wells of the lipid plate and mix. This resulted in the final concentrations of cationic lipid and DNA prior to transfection as indicated below. The molar ratio were as follows:

		1	2	3	4	5	6	7	8	
15	A	168.00 60.60 2.77	84.00 60.60 1.39	42.00 60.60 0.69	21.00 60.60 0.35	10.50 60.60 0.17	5.25 60.60 0.09	2.63 60.60 0.04	1.32 60.60 0.02	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
20	B	168.00 30.30 5.55	84.00 30.30 2.77	42.00 30.30 1.39	21.00 30.30 0.69	10.50 30.30 0.35	5.25 30.30 0.17	2.63 30.30 0.09	1.32 30.30 0.04	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
25	C	168.00 15.15 11.09	84.00 15.15 5.55	42.00 15.15 2.77	21.00 15.15 1.39	10.50 15.15 0.69	5.25 15.15 0.35	2.63 15.15 0.17	1.32 15.15 0.09	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
30	D	168.00 7.57 22.19	84.00 7.57 11.09	42.00 7.57 5.55	21.00 7.57 2.77	10.50 7.57 1.39	5.25 7.57 0.69	2.63 7.57 0.35	1.32 7.57 0.17	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
35	E	168.00 3.79 4.33	84.00 3.79 22.19	42.00 3.79 11.09	21.00 3.79 5.55	10.50 3.79 2.77	5.25 3.79 1.39	2.63 3.79 0.69	1.32 3.79 0.35	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
40	F	168.00 1.90 88.41	84.00 1.90 44.33	42.00 1.90 22.19	21.00 1.90 11.09	10.50 1.90 5.55	5.25 1.90 2.77	2.63 1.90 1.39	1.32 1.90 0.69	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
45	G	168.00 0.95 176.84	84.00 0.95 88.42	42.00 0.95 44.33	21.00 0.95 22.19	10.50 0.95 11.05	5.25 0.95 5.55	2.63 0.95 2.77	1.32 0.95 1.39	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio
	H	168.00 0.48 353.68	84.00 0.48 176.84	42.00 0.48 88.42	21.00 0.48 44.33	10.50 0.48 22.19	5.25 0.48 11.09	2.63 0.48 5.55	1.32 0.48 2.77	[Lipid]( $\mu$ M) [DNA]( $\mu$ M) L/DNA ratio

Column 9 and column 10 (not shown) were DNA control and lipid controls respectively. Column 11 and 12 (not shown) were controls without any lipid. Media was aspirated from the cells and 100 ul of cationic lipid/DNA complex was immediately added to the cells. The plates were incubated at 37°C in a humidified, 5 to 10% CO<sub>2</sub> environment. 50 ul of 30% BCS (bovine calf serum) in OPTI-MEM was added 4-6 hours post-transfection. If the cells are transfected soon after tissue dissociation, and the transfection method employs cationic lipid, then Opti-MEM I (Gibco) media or a similar product is

recommended. During the transfection step Opti-MEM I provides good cell viability under the reduced serum conditions required for Lipofectin™.

5 Incubation was continued and an additional 100 ul of 10% FCS in OPTI-MEM is again added 24 hrs post-transfection. An example of data analysis from this optimization procedure is described in Example 2.

#### mRNA Transfection

10 The procedures required for RNA transfections are identical to the DNA methodology, with the exception that the optimum quantity of Lipofectin™ required per unit mass of RNA will typically be somewhat less than that for DNA transfections and the harvest time for gene expression is 8 hours after addition of the message. These details may be  
15 worked out using the methods described above, substituting RNA for DNA. It is important to recognize that messages that are translatable in in vitro reticulocyte lysate systems, may not be active on intact cells. Intracellular activity requires message that is flanked by untranslated elements.  
20 B. Malone et al. (supra) describes an exemplary luciferase message which contains the 5' and 3' untranslated regions present in the Xenopus beta globin mRNA as well as a 3' poly A tail and a 5' Me-G cap. All of these untranslated elements were shown to be necessary for significant expression in  
25 intact cells.

#### EXAMPLE 2

30 Method for Determination of beta-galactosidase Activity in the 96-well Format.

48 hours after transfection in the 96 well plates, the cells were lysed with 50 ul lysis buffer (0.1% Triton X-100, 250 mM Tris pH 8.0) per well. The plate was frozen at -70°C  
35 before assay. 50 ul of PBS containing 0.5% BSA was added to each well except for column 12. Serial dilutions of beta-galactosidase standard were prepared in PBS containing 0.5% BSA and added to the last column. (eg. 50 ul of 50000, 25000,

12500, 6250, 3125, 1562, 781, 390 pg/ml standards). 150 ul substrate was added to the wells. The substrate consisted of either 1 mg/ml CPRG (chloroform red galactopyranoside) or 2 mg/ml of ONPG (ortho-nitrophenol galactopyranoside) in beta-gal buffer. Beta-gal buffer contains 60 mM sodium dibasic phosphate buffer, pH 8, 1mM magnesium sulfate, 10 mM potassium chloride and 50mM betamercaptoethanol. The substrate was added to each well and the results were read at 580 nm for the CPRG assay or 405 nm for the ONPG assay on a microtiter reader after color development. The CPRG assay was more sensitive than the ONPG and was also less affected by light scattering from cell debris. The assay can also be performed using an alkaline phosphate reporter gene with p-nitrophenol phosphate as a substrate. In addition it can be adapted to an ELISA format by transferring a portion of the cell lysate to antibody coated plates.

### EXAMPLE 3

Basic Assay Characteristics for Cos 7 cells using a 1:1 mixture of DORI and DOPE

The data in Figure 1 display the outcome of a typical transfection assay using this procedure. The bars on the 3D plot represent the level of beta-galactosidase expression in individual wells of the microtiter plate. The cells in each well were treated differently with respect to the levels of DNA and cationic lipid present in the well. Along one axis of the two dimensional matrix are shown serial dilutions of cationic lipid, and the other axis has serial dilutions of DNA. Optimal activity occurs near the middle of the plate. The decline in activity at high cationic lipid concentrations is attributed to toxicity of the lipid; this is supported by direct observations of the cells under the microscope which show that the cells can be severely affected by high concentrations of lipid. The DNA and cationic lipid concentrations must be simultaneously varied in order to obtain optimal transfection activity. A balance of cationic

lipid and polynucleotide is required for efficient delivery since cationic lipid can be toxic to cells at high concentrations.

5 The data in Figure 2 illustrates the effect of changing cell concentration within a microtiter well. As the number of cells per well increased the maximum level of expression per well also increased and the region of maximal expression shifted to higher cationic lipid concentrations. Visual observations of the cells indicated that the cationic lipid was more toxic to the cells at lower cell densities. Thus  
10 cationic lipid toxicity is a function of the amount of lipid per cell. At very high cell densities a plateau in the optimal level of expression appears to have been reached. This data also indicates that smaller numbers of cells could be efficiently transfected in vessels smaller than the size  
15 of a 96-well microtiter plate given that since the amount of lipid per cell could be varied accordingly. Therefore, small numbers of cells preferably 50-100 cells/well could be efficiently transfected in wells preferably having diameters  
20 of 1 to 3 mm.

A comparison of transfection activity among different cell types reveals a substantial variation in the maximum levels of protein expression. Mouse L cells produced 400 fold less gene product than BHK cells. The differences in  
25 transfection efficiencies may be due to the efficiency of cationic lipid mediated cell fusion. There may be differences in intrinsic intracellular transcriptional and/or translational efficiencies among different cell types. The intracellular polynucleotide degradation capacities may vary, or uptake from the cytoplasm into the nucleus may differ.  
30 Three different mouse muscle cell lines were successfully transfected at the same level as COS7 cells and primary rat brain astrocytes were also transfected at these concentrations. These results emphasize the importance of  
35 optimizing the transfection process for each different cell type and vector as outlined in Examples 1 and 2. Thus an

effective amount of lipid will be one determined for each application using the optimization protocols provided herein.

## EXAMPLE 4

5

Lac Z expression in COS-7 cells replaced in vivo.

10

The following procedure is provided for a sample size of 20,000 cells using a DNA plasmid vector pSVZ containing the gene for Lac Z along with the necessary SV40 regulatory elements to promote gene expression (modified pSV plasmid from Pharmacia, Piscataway NJ).

15

Cationic lipid vesicles of a 1:1 mixture of DORI/DOPE were diluted to 0.747 mM in sterile water. 20,000 COS 7 cells/well were seeded in a 96 well microtiter plate. In a second plate 60 ul Opti-MEM with 54 ul of 0.747 cationic lipid was combined. Serial two-fold dilutions were prepared in Opti-MEM to obtain a final cationic lipid concentration of 84 uM. The DNA was serially diluted in Opti-MEM to a final concentration of 60 uM. Equal volumes of cationic lipid and DNA were mixed to produce a final concentration of 42 uM lipid and 30 uM DNA to achieve a Lipid:DNA ratio of 1.39. The media or balance saline was removed from the cells and 100 ul of the cationic lipid/DNA complexes was added to the cells. The cells were incubated at 37°C in 10% CO<sub>2</sub> for 4-6 hours. Following transfection, the cells were washed. These cells are then injected subcutaneously into african green monkeys. Forty-eight hours later the tissue is removed and sampled for beta-galactosidase activity.

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25  
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-30-

## EXAMPLE 5

Ex Vivo IgG Antibody Titer

5           In this ex vivo, strategy for eliciting in vivo antibody  
titers (Figure 3), a single 60 mm tissue culture plate,...  
containing 1 million NIH 3T3 fibroblasts, was transiently  
transfected with 10 µg of plasmid DNA encoding the HIV gene  
gp120 using cationic lipids (DORIE/DOPE) to introduce the DNA  
10       into the cells. A pBR322 based plasmid (Pharmacia) was  
modified to include an RSV promoter and included the HIV  
gp120 gene. Forty hours after transfection the cells were  
washed, removed from culture and injected into syngeneic  
Swiss NIH mice. IgG anti-gp120 antibodies were detected 12  
15       days after injection and levels peaked 28 days post injection  
using a standard Elisa known to those of skill in the art and  
employing HIV infected H9 cell lysates. Antibody titers were  
relatively high with an average serum titer of 1/20,000. The  
cells were transiently transfected with the gene for the  
20       antigen. No prolonged tissue culture selection was required  
to produce the observed humoral response. Animals may  
require a second injection of plasmid to boost the immune  
response (see example 6); however a boost was not required  
for a high titered response to gp120 in mice.

25

## EXAMPLE 6

Ex Vivo Immunization for Schistosomiasis

30

          In this example, two plasmids are prepared using a  
pBR322 based plasmid with RSV regulatory elements linked to  
either luciferase (control) or the immunodominant fragment of  
Schistosome protein Sj23 (Wright et al., J. Immunol.  
35       147:4338-4342, 1991 and Davern et al., Mol. Biochem.  
Parasitol. 48:67, 1991, hereby incorporated by reference).  
Plasmid DNA containing polynucleotide encoding Sj23 is  
prepared in a 1:1 mixture of DORI/DOPE. An equivalent  
concentration of 150 µg plasmid DNA with cationic liposomes



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is bilaterally injected into each thigh of Balb/C mice. One half of the mice receiving pRSV-luciferase control and the other half receiving pRSV-Sj23. A second vaccination into one thigh is repeated twenty-two days after primary injection. Schistosoma mansoni is used to challenge the animals 12 days after the second injection. Animals are bled weekly for one month following the challenge. Titers are compared between the two animals to determine whether the antibodies generated from the immunodominant region of the schistosome protein protect against virus challenge.

## EXAMPLE 7

Polynucleotide delivery to Myoblasts Ex Vivo

Example 7 illustrates a method to express exogenous polynucleotide sequences in muscle cells. In this example myoblasts are treated with a gene of interest and introduced back into whole muscle.

The mouse myoblast cell lines used in the study were C2, G7, and G8. C2 is a mouse myoblast cell line established from normal adult C3H mouse leg muscle (Nature 270: 725-727; Science 230: 758-766, hereby incorporated by reference). G7 and G8 are mouse myoblast cell lines isolated from the hind limb muscle of a fetal Swiss Webster mouse. G7 is tumorigenic whereas G8 is non-tumorigenic (Science 196:995-998, 1977, hereby incorporated by reference). To determine the feasibility of using these muscle cell lines, transient transfections were performed using the pSV2-LacZ reporter construct with DORIE/DOPE concentrations optimized from Example 1. These cells were implanted into the hind limb of Balb C mice. Tissue sections from injected muscle are stained using antibody to LacZ. Similarly, blood samples are assayed by ELISA for LacZ protein. All transfected cells are tissue positive for LacZ and myoblast cell lines G7 and G8 are additionally serum positive. The results indicate that

the myoblasts can be transfected in vitro using methods applicable to other commercially available cell lines.

## EXAMPLE 8

5      Delivery of Human  $\alpha_1$ -antitrypsin to an Animal by in vitro  
Transfection and Replacement of Autologous Hepatocytes

Hepatocytes are isolated from a liver sample obtained by partial hepatectomy, placed in culture medium and returned to  
10      the animal after transfection essentially as described by Kay, M. et al., Proc. Natl. Acad. Sci USA 89:89-93 (1992).

A quantity of from 5000 to 20,000 hepatocytes, isolated by colleganase treatment from a liver sample taken from an animal subject, are seeded onto each well of a 96-well  
15      microtiter plate and transfected according to the procedures described in Examples 1, 2 and 4. Cationic lipids for transfection are a 50:50 mixture of DORI:DOPE, and cells are transfected by means of a DNA plasmid vector pSVZ construct, comprising the human  $\alpha_1$ -antitrypsin cDNA under  
20      transcriptional control of the cytomegalovirus promoter. Concentrations and ratios of transfection lipids and DNA are determined according to the scheme of Example 2. Following incubation at 37°C in 10% CO<sub>2</sub> for 4-6 h, the transfected cells are rinsed in basal media comprising 2-10% animal  
25      serum, and resuspended in the same solution at a concentration of 10<sup>6</sup>-10<sup>7</sup> cells/ml. The solution containing the transfected cells is then injected into the portal circulation of the liver of the same animal, preferably by means of an infusion pump at a rate of about 0.5 to 2.0  
30      ml/min. The in vivo secretion of human  $\alpha_1$ -antitrypsin is followed by assay of serum levels of human  $\alpha_1$ -antitrypsin.

## EXAMPLE 9

Delivery of Human Nerve Growth Factor to the Brain Tissue  
of an Animal by in vitro Transfection and Replacement of  
Autologous Astrocytes

5

Brain cells are isolated from a biopsy specimen taken from the region of the brain of an animal, placed in culture medium and returned to the animal after transfection with cDNA for nerve growth factor, essentially as described in Example 8 above.

10

The cells are transfected by means of a DNA plasmid vector pRSV, pSV2, or pCMV construct, comprising the human nerve growth factor cDNA under transcriptional control of the early SV40 or immediate early CMV promoters. Following incubation at 37°C in 10% CO<sub>2</sub> for 4-6 h, the transfected cells are rinsed in basal media, and resuspended in the same solution at a concentration of 10<sup>6</sup>-10<sup>7</sup> cells/ml. The solution containing the transfected cells is then injected back into an appropriate brain site for therapy. In animal subjects, a repeat biopsy is used to confirm expression. Where the repeat biopsy is inappropriate, a sample of cells is retained for culture to confirm expression. Expression of nerve growth factor is demonstrated by an Elisa procedure using monoclonal antibody to nerve growth factor as a binding agent.

15

20

25

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined in the following claims.

30

WHAT IS CLAIMED IS:

1. A method for preparing transfected cells for use expressing a polypeptide in a vertebrate, comprising the steps of:

5 removing live cells from said vertebrate; and  
transfecting at least some of said live cells  
by contacting them with a preparation comprising  
an effective amount of a polynucleotide  
operatively encoding said polypeptide and a  
10 transfection-facilitating lipid, said preparation  
being effective to deliver a substantial amount of  
said polynucleotide into said cells by contact  
therewith within 48 hours; and

15 suspending said transfected cells in a  
pharmacologically acceptable carrier for introduction  
into said vertebrate, in the absence of a selection step  
to separate or expand said transfected cells.

2. The method of Claim 1, wherein said removing step  
20 additionally comprises substantially separating said cells  
from the surrounding extracellular matrix.

3. The method of Claim 1, wherein said cells are  
frozen within 48 hours after the contacting step.

4. The method of Claim 1, wherein said peptide is  
25 adapted to treat a disease of said vertebrate caused by a  
functional gene deficiency.

5. The method of Claim 1, wherein said polynucleotide  
is adapted to express said polypeptide in said live cells.

6. The method of Claim 5, wherein expression of said  
polynucleotide by said cells is transient.

30 7. The method of Claim 5, wherein said polypeptide is  
an immunogenic polypeptide in said vertebrate.

8. The method of Claim 5, wherein said polynucleotide  
operatively codes for a lymphokine.

35 9. The method of Claim 5, wherein said cells are white  
blood cells.

10. The method of Claim 5, wherein said cells are bone  
marrow cells.

11. The method of Claim 7, wherein said cells are myoblasts.

12. A medicament prepared in accordance with any one of Claims 1-11.

5 13. A method for delivering a polypeptide to a vertebrate *in vivo*, comprising the step of injecting or implanting the medicament of Claim 12 into said vertebrate.

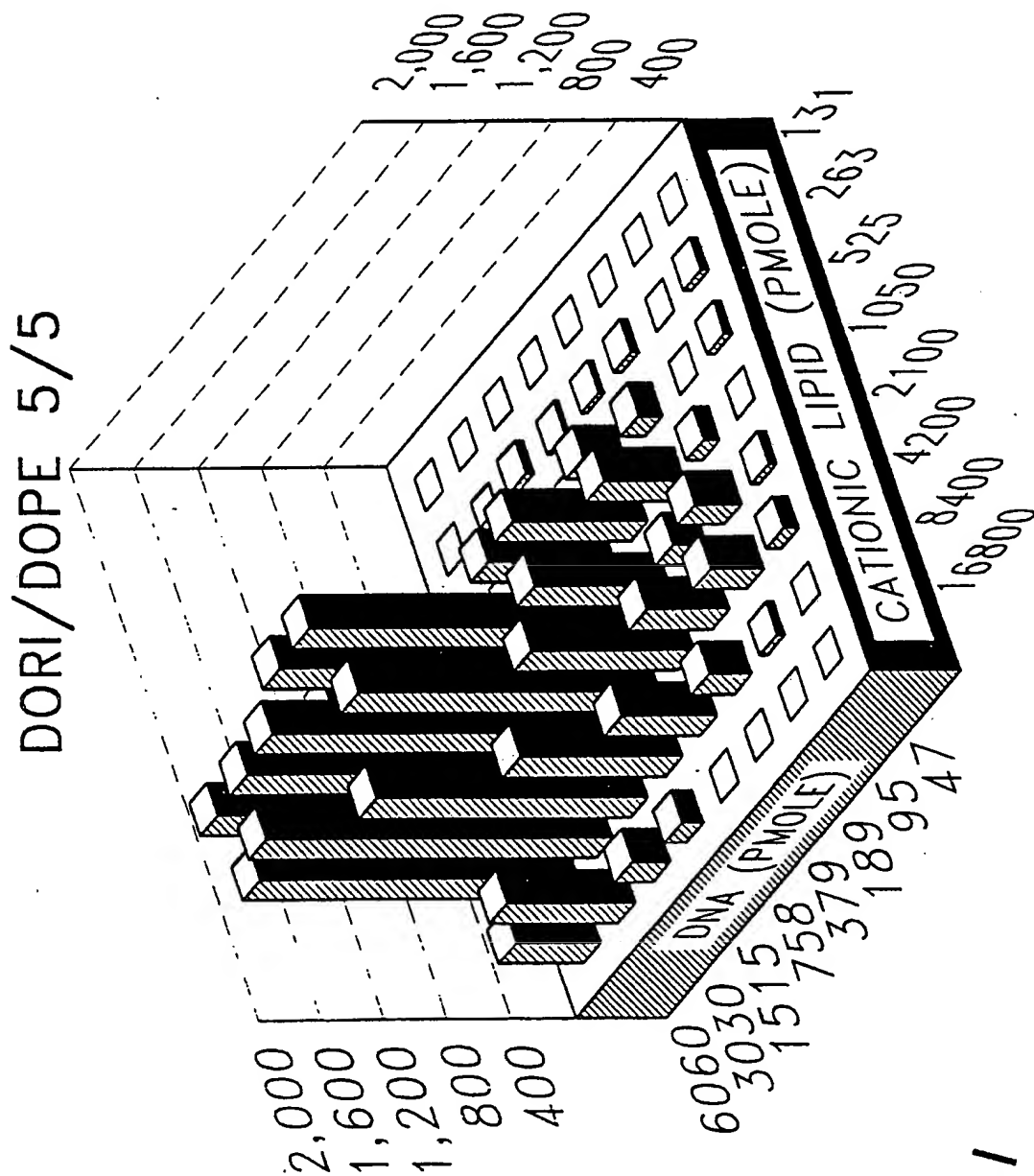


FIG. 1

Pg EXPRESSION OF BETA-GAL: pSV2-lacZ ON COS.7

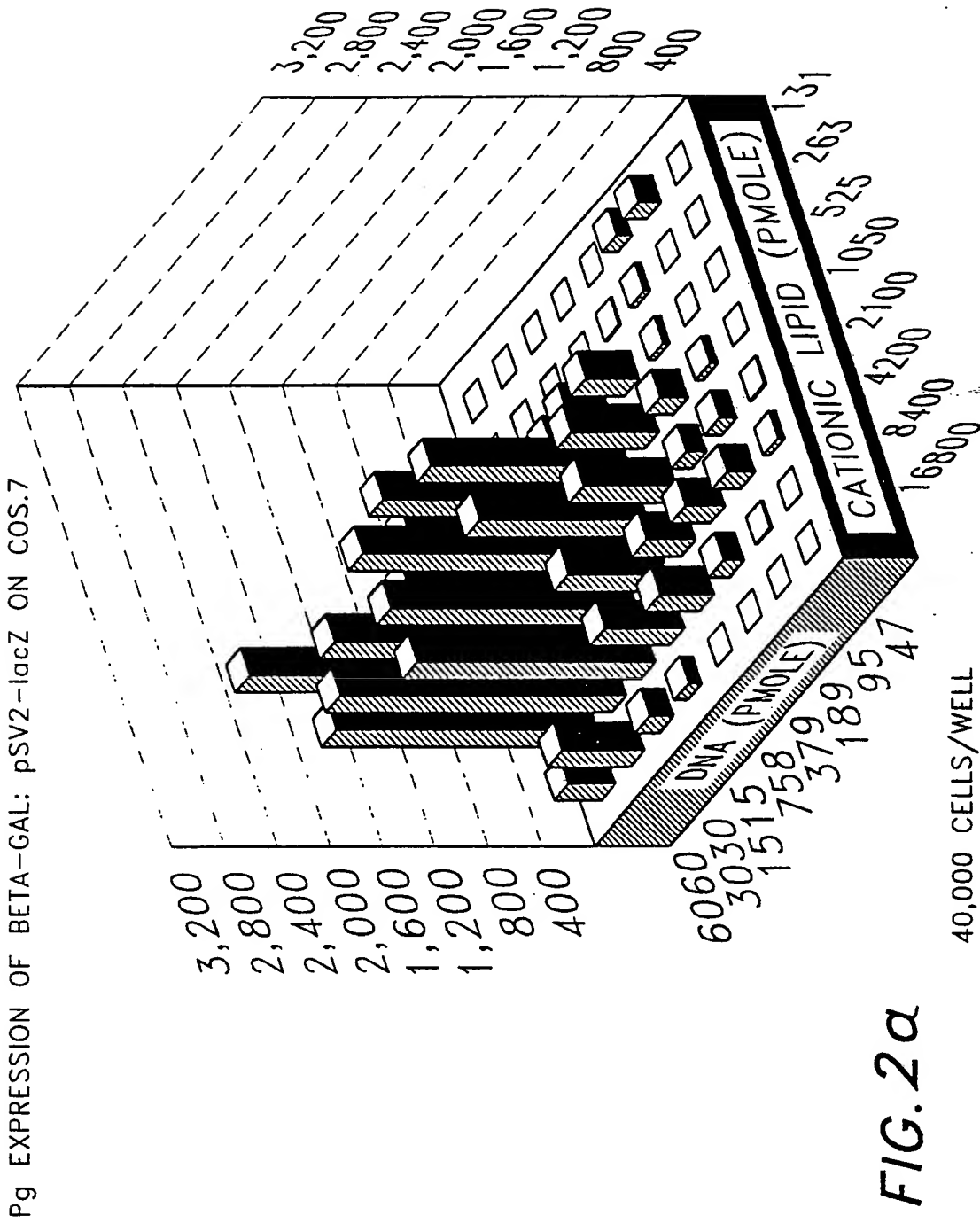
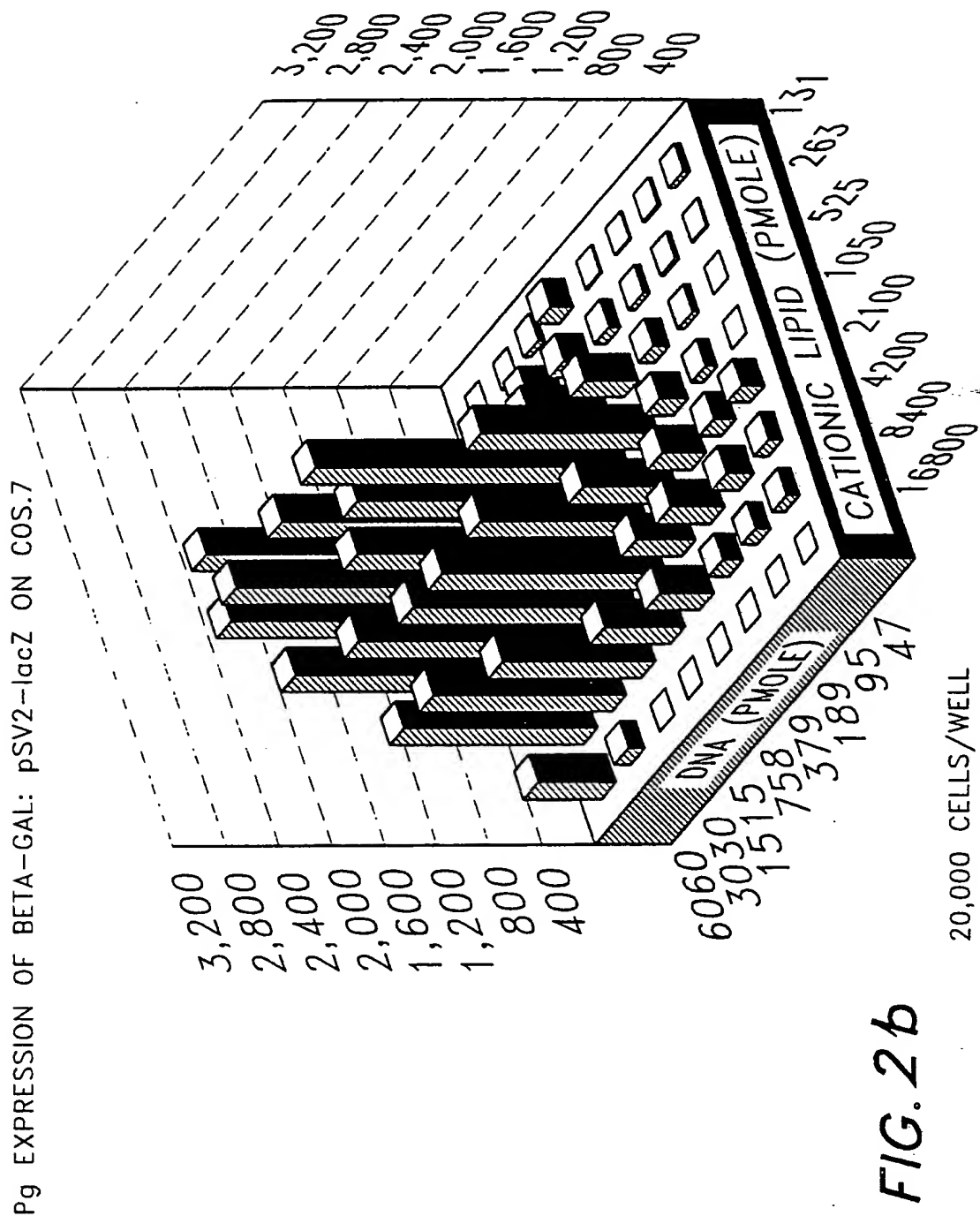


FIG. 2a





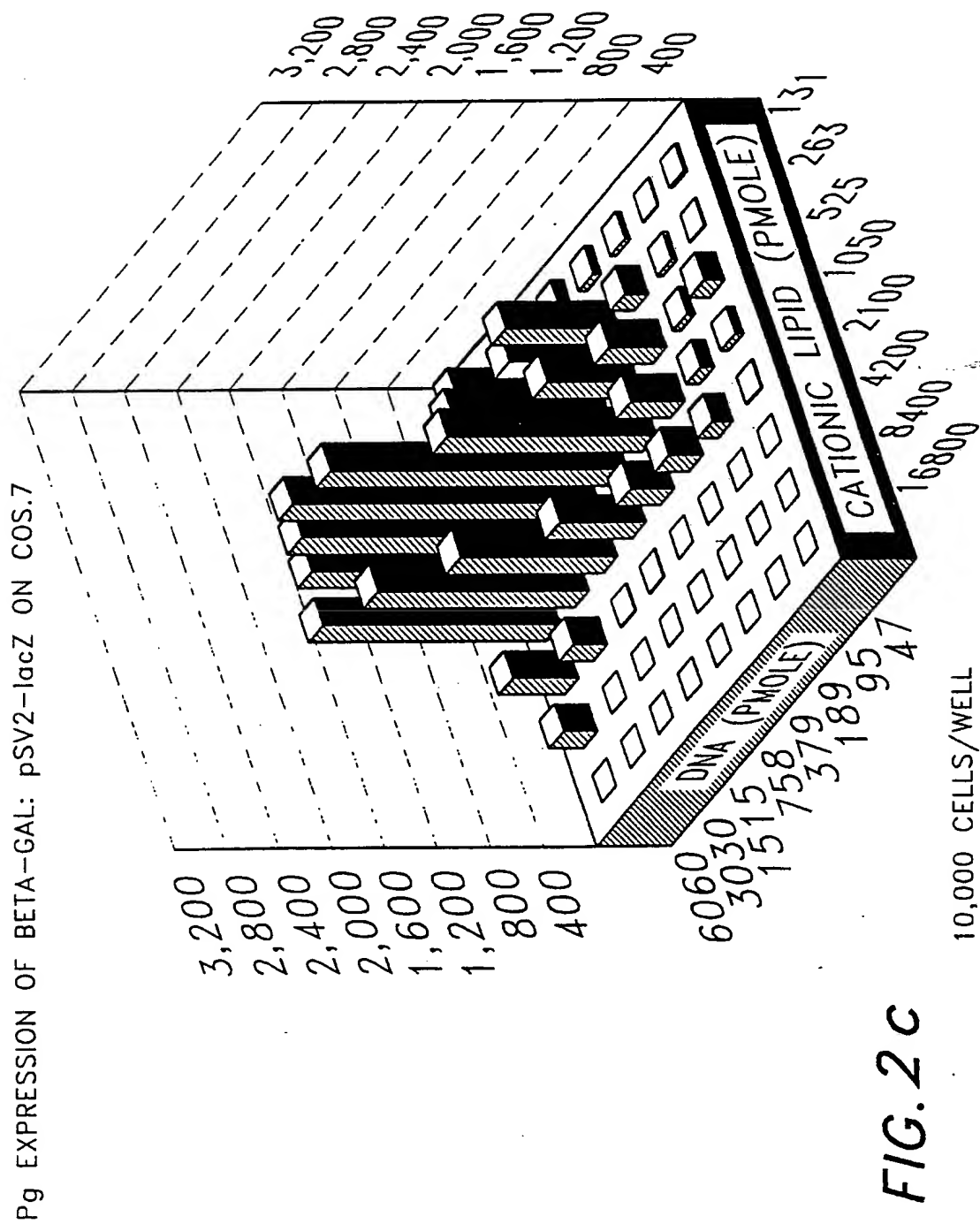


FIG. 2 C

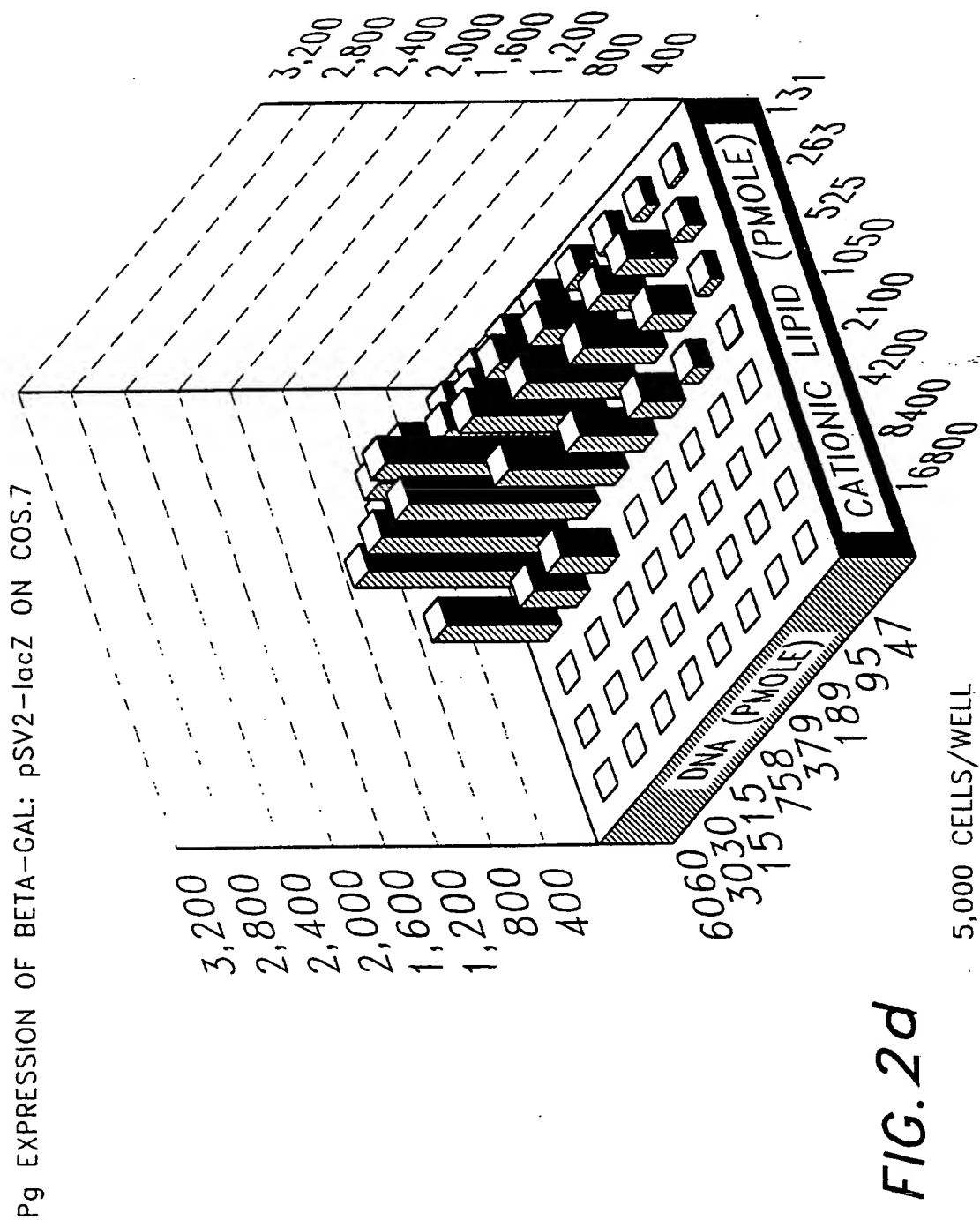


FIG. 2d

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EX VIVO IMMUNIZATION  
IgG ANTIBODY TITER

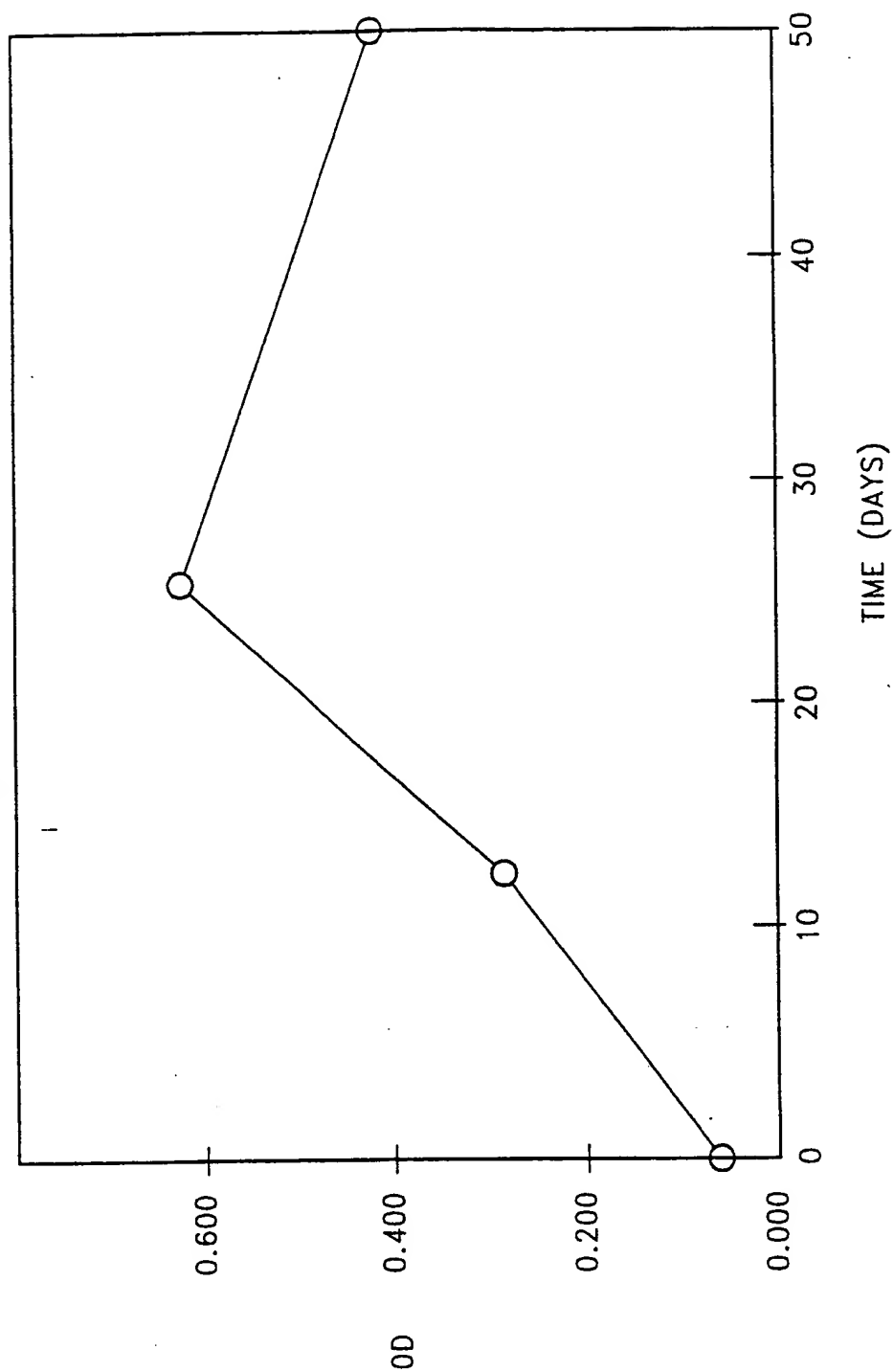


FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00492

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00

US CL :424/93

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93, 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, BIOGES, MEDLINE , - "Gene therapy" Lipofechi", somati cell gene therapy"

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biotechniques, volume 6, no. 7, issued 1988, Mannino <u>et al.</u> , "Liposome Mediated Gene Transfer", pages 682-690, see entire article.	1-4
Y	Science, volume 254, issued 06 December 1991, Barr <u>et al.</u> , "Systemic Delivery of Recombinant Proteins by Genetically Modified Myoblasts", pages 1507-1509, see entire article.	1-4, 7-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

30 MARCH 1993

Date of mailing of the international search report

12 MAY 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

SUZANNE ZISKA

VSH

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/00492

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, volume 86, issued November 1989, Wolff <u>et al.</u> , "Grafting Fibroblast genetically modified to produce L-dopa in a rat model of Parkinson disease", pages 9011-9014, see entire article.	4-6, 12, 13
Y	Cancer Research, volume (supplement) 51, issued 15 September 1991, Rosenberg <u>et al.</u> , "Immunotherapy and Gene Therapy of Cancer", pages 5074s-5079s, see entire article.	8
Y	Molecular Cellular Biology, volume 4, no. 6, issued June 1984, Kawai <u>et al.</u> , "New Procedure for DNA transfection with Polycation and Dimethyl Sulfoxide", pages 1172-1174, see entire article.	7-11
Y	Proceedings of the National Academy of Sciences, volume 85, issued May 1988, St. Louis <u>et al.</u> , "An Alternative approach to somatic cell gene therapy", pages 3150-3154, see entire article.	7-11